CHEMISTRY OF THE SHEATH OF THE CYANOBACTERIUM
LYNGBYA AESTUARII LIEB.

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Dedicated to the memory of Professeur Pierre Bourrelly

ABSTRACT — The sheath of the cyanobacterium *Lyngbya aestuarii* has been shown to be a sulfated proteoglycan. The polypeptide comprises 12.9% of the sheath dry weight and sulfate esters account for 2.0%. Aspartic acid and alanine represent 32.5% of the polypeptide component. The carbohydrate moiety contains arabinose, galactose, glucose, mannose, rhamnose, xylose and the uronic acids, galacturonic and glucuronic acid. The dominant monosaccharide is glucose, averaging 18.0% of the dry weight. At least 13 different monosaccharide linkages have been identified. The data from these analyses suggest that this morphologically rigid sheath is a single sulfated proteoglycan. The ultraviolet shielding pigment scytonemin, while located in the sheath, is not an integral part of the sheath. Scytonemin is easily removed by solvent extraction and there are no significant differences in the composition of pigmented and unpigmented sheaths.

RÉSUMÉ — Cette étude montre que la gaine de la cyanobactérie *Lyngbya aestuarii* est un protéoglycane sulfaté. Le polypeptide représente 12,9% de la masse sèche de la gaine et les ester-sulfate 2,0%. Il est composé pour 32,5% d'acide aspartique et d'alanine. La fraction hydrocarbonée contient de l'arabinose, du galactose, du glucose, du mannose, du rhamnose, du xylose, de l'acide galacturonique et de l'acide glucuronique. L'ose dominant est le glucose qui représente en moyenne 18,0% de la masse sèche. Des liaisons entre, au moins, 13 oses différents ont été identifiés. Les données de ces analyses suggèrent que cette gaine morphologiquement rigide est constituée d'un seul protéoglycane. Le pigment protecteur contre les ultra-violets, la scytonémine, bien que située dans la gaine, n'en est pas partie intégrante. La scytonémine est aisément extraite à l'aide d'un solvant et il n'existe pas de différences significatives entre les compositions des gaines pigmentées et dépigmentées.

KEY WORDS: carbohydrates, Cyanobacteria, *Lyngbya*, marine microalgae, protein, sheath, scytonemin.

INTRODUCTION

Lyngbya aestuarii is an unbranched filamentous cyanobacterium belonging to Section III of the cyanobacteria complex according to the classification of Rippka, *et al* (1979). A filament of *L. aestuarii* is composed of a trichome of disc-shaped cells surrounded by a 1-3 μm thick firm fibrillar sheath (Lang, 1968). The trichome has the ability to move through the cylinder of the sheath, principally in response to light. Empty sheaths, lacking trichomes, retain their shape and are often observed in cultures. *L. aestuarii* typically grows as a felt-like mat of intertwined filaments in the intertidal zone of marginal marine and coastal hypersaline environments. Both morphology and growth habit promote trapping of sediment particles, which may also be bound to the sheath surface. Thus, net accretion and preservation of stromatolitic material may occur under favorable environmental conditions (Bauld, 1981).

Under field conditions the habitat of *L. aestuarii* mats receives intense solar radiation and sheaths at the surface of the mat become pigmented with scytonemin, a yellow-brown pigment, which is deposited in the sheath. The sheaths just below the surface layer remain unpigmented. The function of this pigmentation as an effective ultraviolet screen has been the focus of ■ series of investigations by Garcia-Pichel & Castenholz (1991) and Garcia-Pichel *et al.* (1992). The chemical structure of scytonemin was determined by Proteau *et al.* (1993).

L. aestuarii bears a close resemblance to Precambrian microfossils with preserved sheath segments from the Early and Late Proterozoic, 2.3 10^8 to 8.5 10^8 years ago, (Schopf, 1968; Klein *et al.*, 1987). Attempts to artificially fossilize *Lyngbya* filaments resulted in artificial fossils which resembled their naturally occurring counterparts (Oehler & Schopf, 1971). Individual cells of the trichome were not preserved but the tubular structure of the sheath was organically preserved and clearly distinguishable. Bartley (1996) confirmed that the sheath of *L. aestuarii* was significantly more resistant to decomposition than the cells of the trichome.

Understanding the preservation properties of *Lyngbya* sheaths requires a knowledge of their chemistry. Since the sheath plays a role in UV protection and shows pigmentation in response to high irradiance, the possibility of chemical variation in the sheath must be considered, together with the question of whether or not scytonemin is an integral part of the sheath, possibly contributing to its structural integrity. This paper describes the chemistry of the sheath of *Lyngbya aestuarii* LM 8701. The data indicate that the sheath chemistry itself is not influenced by irradiance levels, and accumulated scytonemin remains chemically independent of the sheath structure.

METHODS

Organism and growth conditions. *Lyngbya aestuarii* Lieb. strain LM 8701 is an isolate collected from Shark Bay in Western Australia by J. Bauld. Axenic cultures were grown in modified Erdschreiber medium formulated as follows: 1 liter of artificial sea water constituted by dissolving 32 g of sea salts (Sigma Chemical Co.) in distilled water and bringing the volume to 1 liter. The following supplements were added: 200 mg

NaNO$_3$, 20 mg K$_2$HPO$_4$, 1 ml Fe-EDTA solution (4 g FeSO$_4$ 7H$_2$O, 5 g Na$_2$EDTA and distilled water to 1 liter), 30 ml PII metal mix [1 g Na$_2$EDTA, 1.1 g H$_3$BO$_3$, 48 mg FeCl$_3$ 6H$_2$O, 144 mg MnCl$_2$ 4H$_2$O, 10.4 mg ZnCl$_2$, 4.3 mg COCl$_2$ 6H$_2$O and distilled water to 1liter (Provasoli *et al.*, 1957)], and 1 ml vitamin solution (500 mg thiamin, 10 mg vitamin B12, 20 mg biotin, 500 mg niacinamide, 100 mg p-aminobenzoic acid and distilled water to 1 liter). Cultures were grown at 25-30° C in 2.8 liter Fernbach flasks stoppered with foam plugs. Continuous irradiance of 15-30 µEm^{-2} sec^{-1} was provided to the cultures by a 15 watt cool-white fluorescent bulb. These culture conditions produced a felt-like mass of filaments characterized by colorless sheaths. Pigmentation of the sheath was induced by placing sections of the cultured mat, with medium, in glass petri dishes under continuous irradiance of 140 µEm^{-2} sec^{-1} produced by a bank of cool-white fluorescent bulbs suspended 10 cm above the culture.

Isolation of sheath. The aggregated mat of filaments of *L. aestuarii* was removed intact from the culture flasks or petri dishes and washed with distilled water. Portions of the mat were first frozen in liquid nitrogen then ground to a powder with dry ice in a pre-cooled electric coffee grinder. Sheath material, free of intact cells, was prepared by the method of Adhikary *et al.* (1986), modified for the high density of the sheath fragments. Aliquots of the powdered sample were suspended in a volume of distilled water equal to 10 × the original wet weight of cell mass and passed through a French pressure cell at 96 MPa. The crude sheath fraction was recovered from the ruptured filaments by low speed centrifugation (15 min at 3,500-5,000 g) in a swinging bucket rotor and washed 4 × with distilled water or until the supernatant was no longer green. The pellet containing the sheath was resuspended in 10 mM Tris-HCl buffer pH 8.0 and treated overnight at 42° C with egg-white lysozyme (EC 3.2.1.17) at an enzyme concentration of 20 mg ml^{-1} to facilitate removal of any residual cell wall from the sheath. The treated sheath fraction was recovered by low speed centrifugation and the pellet containing the sheath was washed 2 x with distilled water. The pellet was resuspended in a solution of 4% w/v sodium dodecyl sulfate (SDS) equal to 15 x the original sample wet weight and placed in a water bath at 100° C for 20 min. The suspension was then diluted with distilled water, to reduce its viscosity, and the pellet recovered by low speed centrifugation. The pellet was washed 5 × with distilled water to remove the SDS. After each wash any cell debris remaining in the supernatant were discarded. A dilute suspension of the washed pellet in distilled water was loaded onto a discontinuous sucrose gradient (50% w/v sucrose over 60% w/v sucrose) and centrifuged at 2,700 g in a swinging bucket rotor for 50 min at 4° C. Purified sheath was recovered as a pellet and washed with distilled water 4 x by low speed centrifugation before lyophilization. Visual observation by light microscopy revealed purified sheath fragments with no residual cellular material.

Scytonemin was extracted from colored sheath material into 90% acetone in water from an aqueous pellet. When acetone was removed under a stream of nitrogen, scytonemin was observed to crystallize and precipitate. Crystals were recovered by centrifugation or by settling overnight. Scytonemin was also extracted from the colored sheath by the method of Garcia-Pichel & Castenholz (1991) using methanol at 40° C followed by the addition of distilled water. Volumes were reduced with a rotary evaporator and crystals collected on GF/F glass fiber filters. Pigment crystals were removed from the filters by dissolving into tetrahydrofuran. The pigment extract was further purified on a Sephadex LH-20 column followed by high pressure liquid chromatography (HPLC) on a normal phase silica gel column (Beckman Instruments).

Analytical methods. Amino acid analysis was performed on a Pico-Tag amino acid analysis system as described by Bidlingmeyer *et al.* (1984). Sheath samples (2-100 µg) were hydrolyzed under vacuum in 6 M HCl at 110° C for 18 hours. Amino acids were also determined with a modified ninhydrin assay (Rosen, 1957). Amino acids were released from the sheath polypeptide by hydrolysis in 6M HCl for 16 hours at 100° C. Monosaccharides were released from sheath samples by hydrolysis in 2 M trifluoroacetic acid (TFA) in a sealed tube under nitrogen at autoclave temperature and pressure for 1, 2 or 3 hours. TFA was removed by reducing the sample to dryness under a stream of dry nitrogen. Initial examination of sheath carbohydrates was conducted by thin layer chromatography on silica gel G plates (solvent: ethyl acetate-pyridine-water; 12:5:3 v/v). The monosaccharides, both neutral sugars and uronic acids, were visualized by the naphthoresorcinol-sulfuric acid reagent No. 175 (Stahl, 1969). Neutral sugars were identified and quantified by gas liquid chromatography: mass spectrometry (GC-MS) of their alditol acetate derivatives (Blakeney *et al.*, 1983; York *et al.*, 1985) using an SP2330 capillary column (0.25 mm × 30 m). The injector and detector were held at 250° C, initial column temperature was 190° C increased at 10° C min⁻¹ to 240° C and held there for 10 min. The alditol acetate derivatives were quantified as µg mg⁻¹ of sample weight using the detector response value for each sugar. Mass spectrometric analysis was performed using a 5970 Mass Selective Detector (Hewlet-Packard) at 70 eV in the mass range m/z 40-550. Trimethyl silyl derivatives of neutral sugars and uronic acids were also prepared (York *et al.*, 1985). Glycosyl linkage analysis was performed by GC-MS of partially methylated alditol acetates (York *et al.*, 1985) using the same column as above, with an initial column temperature of 80° C increased at 30° C min⁻¹ to 170° C then increased at 4° C min⁻¹ to 240° C and held there for 10 min. Uronic acids were quantified using the method of Blumentkrantz and Asboe-Hansen (1973). Glucose was also measured enzymatically with a β-D-glucose oxidase as described by Sturgeon (1990). Glucose was released from the sheath by TFA hydrolysis for 1, 2 or 3 hours or by hydrolysis in 1 M HCl at 100° C for 16 hours in a sealed tube flushed with nitrogen. Analysis of sulfate content was conducted using the method of Terho & Hartiala (1970).

RESULTS

The procedure used for the isolation of the sheath yielded a purified sheath fraction showing no contamination by cell wall fragments when examined under the light microscope. Diaminopimelic acid, a marker for cell wall contamination, was not detected during amino acid analysis where the lower limit of sensitivity was approximately 0.01% of sample analyzed. Approximately 0.6% of the original wet weight of cultured material was recovered as dry weight of purified sheath. However, this is almost certainly a low value since the centrifugation steps in the purification sometimes resulted in the discard of sheath material.

The polypeptide content of pigmented and unpigmented sheath is expressed in terms of both mole % amino acids and total protein present as a percent of dry weight. The averaged amino acid analysis data from pigmented sheath and from unpigmented sheath (Table 1) show great similarity. Pigmented sheath contained 10.7% protein, by colorimetric assay, while unpigmented sheath contained 14.5% protein. Total percent protein as a summation of µg of each amino acid from amino acid analysis of unpigmented sheath

equaled 15.7%. The average total percent protein of both pigmented and unpigmented sheath is 12.9% of dry weight. The amino acid composition of *L. aestuarii* is unremarkable except that aspartic acid and alanine values are both high as compared to the sheaths of other cyanobacteria for which amino acid compositions are available. These two amino acids, aspartic acid (with a small polar side chain) and alanine (with a small non polar side chain), constitute 32.5% of the amino acid composition of the sheath.

Table 1. Amino acid composition of pigmented and unpigmented sheath, as mole %, from *Lyngbya aestuarii* Lieb. strain LM 8701. Sheath samples (2-100 µg) were hydrolyzed in 6 M HCl at 110° C for 18 hours under vacuum. Analyses were performed on a Pico-Tag amino acid analysis system (Bildingmeyer *et al.*, 1984). Total protein was also determined as a % of dry weight using a modified ninhydrin assay. n is the number of separate sheath preparations which were averaged to yield the amino acid composition data presented.

	Pigmented (n = 2)	Unpigmented (n = 3)
Asp	16.5 mole %	15.9 mole %
Glu	8.4	6.8
Ser	9.0	9.4
Gly	7.6	9.1
His	0.3	0.4
Arg	2.2	3.1
Thr	9.5	9.7
Ala	16.6	16.3
Pro	2.7	2.5
Tyr	1.2	1.4
Val	5.7	5.6
Met	0.6	0.8
Ile	4.6	4.5
Leu	5.8	5.9
Phe	5.6	4.7
Lys	4.2	5.1

Carbohydrates in the sheath of *L. aestuarii* were initially identified using TLC methods. With the exception of galacturonic acid, all sugars detected by TLC were also identified when sheath samples were subsequently analyzed by GC-MS. Only one uronic acid, glucuronic, was identified during GC-MS analyses. However, on thin layer chromatograms two uronic acids, glucuronic and galacturonic, were identified by comparison against authentic standards. Why galacturonic was not detected during GC analysis has not been resolved. Recovery of uronic acids from polysaccharides can be variable after reduction for GC-MS (Quintero *et al.*, 1989).

Quantitative estimates for the various monosaccharides were obtained by GC (Table 2). Glucose in the sheath was also quantified with an enzymatic assay using ß-D-glucose oxidase which is specific for ß-D-glucose. Sheath samples were hydrolyzed in the same manner as was used for sample preparation for GC analysis (2 M TFA at autoclave temperature and pressure for 1 hour). The glucose concentration, when measured by using the enzymatic assay, was equivalent to 16.2% of the dry weight of pigmented

sheath and 20.7% of the dry weight of unpigmented sheath. An alternative hydrolysis in
1 M HCl for 16 hours at 100° C yielded glucose concentrations equivalent to 16.3% of the
dry weight of pigmented sheath and 18.8% of the dry weight of unpigmented sheath.
From these data, an average of 18.0% of the sheath dry weight is glucose. When the time
of hydrolysis, using unpigmented sheath, in 2 M TFA at autoclave temperature and
pressure, was extended to 2 and 3 hours, samples yielded glucose concentrations equiva-
lent to 17.4% and 16.8% of sheath dry weight respectively.

Table 2. Quantification of monosaccharides by GC in pigmented and unpigmented sheath of *Lyngbya aestuarii* Lieb. strain LM 8701. Samples were hydrolyzed in 2 M TFA in a sealed tube under nitrogen at autoclave pressure for 2 hours. Monosaccharides were chromatographed on a SP2330 capillary column (0.25 mm × 15 m) as alditol acetate derivatives. The injector and detector were held at 250° C, initial column temperature was 190° C, increased at 10° C min⁻¹ to 240° C, then held for 10 min. Alditol acetate derivatives were quantified as µg mg⁻¹ of dry sample weight. Glucose and uronic acids were also measured using enzymatic and colorimetric assays respectively.

Glycosyl Composition	Pigmented	Unpigmented
Rhamnose	3.2 %	4.5 %
Fucose	0.81	0.67
Arabinose	2.7	1.8
Xylose	2.3	2.3
Mannose	4.3	6.1
Galactose	5.2	7.2
Glucose	8.8	15.0
Glucuronic acid	2.4	5.3

Glucuronic acid when quantified by GC represented 5.3% of the dry weight of
unpigmented sheath. Total uronic acids, as a class, were also quantified in a colorimetric
assay. When measured in glucuronic acid units unpigmented sheath contained 9.1% uronic
acids and when measured in galacturonic acid units it contained 10.4% uronic acids
(glucuronic and galacturonic acids produce slightly different amounts of chromogen in
this assay). An average of 9.8% of the sheath of *L. aestuarii* is found to be uronic acids
when quantified using this colorimetric assay.

The complexity of the polysaccharide sheath of *L. aestuarii* is evidenced by the
number of identified linkages. Linkage analysis of the carbohydrates present in the sheath
was performed by GC-MS. Linkage analysis of partially methylated alditol acetate derivatives.
Glucose, the most abundant sheath carbohydrate, which constitutes an average of 18% of
the sheath dry weight when measured enzymatically, participates in 6 different linkage
positions: Terminal-glucose, 3-glucose, 2-glucose, 4-glucose, 3,6-glucose, and 4,6-glucose.
Galactose, the second most abundant sheath carbohydrate, representing an average of
6.2% of sheath dry weight was identified in 2 linkages: 3,4-galactose and 2,3-galactose.
Mannose, which makes up an average of 5.2% of sheath dry weight was identified in 3
linkage positions: 6-mannose, 3,4-mannose and 4,6-mannose. Rhamnose, which on ave-
rage makes up 3.9% of sheath dry weight, and arabinose, which on average constitutes
2.3% of sheath dry weight, were each identified in a single linkage position: 4-rhamnose
and terminal-arabinose. No linkage positions were identified which involved xylose, which
makes up 2.3% of sheath dry weight on average or fucose which makes up 0.7% of sheath

dry weight on average. However, these sugars must participate at least as terminal sugars. By adding one linkage position for each of these sugars, the total number of different linkages present is brought to fifteen.

DISCUSSION

The possibility that the sheath consists of more than one peptidoglycan or carbohydrate moiety cannot be resolved at this time. The complete insolubility of the sheath and inability to produce smaller soluble derivatives (Robbins, 1992) precludes any chromatographic or electrophoretic separation. Attempts to dissolve the sheath using 8M urea, phenol, formic acid, detergents or ion chelators did not produce any visible change in the sheath fragments or solubilization, therefore no chromatographic separation of sheath fractions was possible. However, the general quantitative reproducibility of individual analyses of sugars and amino acids from sheath of cultures of different age and different growth conditions (Robbins, 1992) suggests that the sheath may be a consistent, if highly resistant, polymer.

The amount of protein associated with untreated sheath varies widely, ranging from a low of 3.6% of dry weight in *Fischerella* sp. (Pritzer *et al.*, 1989) to a high of 22.6% in *Chlorogloeopsis* (Schrader *et al.*, 1982). It has not been possible to remove the protein associated with the purified sheath fraction by treatment with SDS or any other detergent, indicating a sheath peptidoglycan.

The carbohydrate and protein data on the composition of the sheath of *L. aestuarii* agree well with that reported by other authors for other cyanobacteria. In general, glucose is the most abundant monosaccharide of the sheath layer and is the most abundant sugar in the sheath of *L. aestuarii*. Several of the species of cyanobacteria in which the sheath layer has been investigated by other authors (Adhikary *et al.*, 1986; Weckesser *et al.*, 1987; Weckesser *et al.*, 1988; Pritzer *et al.*, 1989) were found to contain O-methyl sugars. However, no O-methyl sugars have been identified in the sheath carbohydrates of *L. aestuarii*. Further complicating sheath composition, an analysis for sulfate revealed a limited amount of sulfate present at 2.0% of total weight. This level of sulfate is similar to that found in *Synechocystis* (Panoff *et al.*, 1988). With the exception of the high alanine and aspartic acid content and the absence of O-methyl sugars the data for *L. aestuarii* are consistent with those from other cyanobacteria.

Analyses of the composition of sheath material are reported on a percent of dry weight basis. When the reported percent dry weight of all the known constituents of the sheath are totaled, they do not equal 100%. The average percent of dry weight which is of known composition is 55%. Thus, 45% of the sheath weight is unaccounted for. In contrast, when the composition of diffuse slime layers was examined the total percent of dry weight, which is of known composition, slightly exceeds 100% (Nakagawa *et al.*, 1987). It is unlikely that sheath material contains an undetected constituent which is responsible for this discrepancy. Instead, this anomaly is probably explained by the resistance of sheath material to hydrolysis under the conditions used during analysis. Weckesser *et al.* (1988) noted that the sheath of *Calothrix parietina* was not completely solubilized during the hydrolysis step prior to analysis.

More severe hydrolysis conditions with increased acid concentration, temperature or length of hydrolysis would be likely to release more monosaccharides from the

sheath polysaccharide. However, a greater loss of released monosaccharides also occurs. The added problem of the varying susceptibilities of these monosaccharides to degradation also exists (Torello *et al.*, 1980). An alternative hydrolysis protocol involving the use of liquid phase anhydrous hydrogen fluoride (Rorrer *et al.*, 1990), was not attempted in our investigation.

The information presented on the linkage structure of the carbohydrates from the sheath of *L. aestuarii* underscores the complexity of the sheath polysaccharide. No other data on linkage of carbohydrates from cyanobacteria sheaths or mucilage have been published to our knowledge.

Attempts to break the sheath carbohydrate into smaller oligosaccharides for detailed analyses were unsuccessful. A number of site specific enzymes (ß-glucuronidase, α-mannosidase) and general mixed enzyme systems (abalone acetone powder, snail acetone powder and Driselase (a mixture of enzymes from the fungus *Irpex lacteus* which contains both endo and exo-hydrolases) failed to produce smaller oligosaccharides under digestive conditions.

The function that proteins play in sheath structure is not known. However, proteins appear to be an intrinsic part of sheath structure. Even after drastic treatments to remove associated proteins (boiling in SDS for 20 minutes) the sheath fraction retained a protein component. This implies the presence of one or more glycoproteins in the sheath. The sheath of *L. aestuarii* contained 12.9% intrinsic protein or glycoprotein and is within the range of values reported in the literature for other cyanobacteria.

Changes in temperature, light intensity and the availability of combined nitrogen have been shown to affect sheath composition or morphology (Findlay *et al.*, 1970; Evans & Foulds, 1976; Tease & Walker, 1989). When *L. aestuarii* was grown under two light regimes to produce pigmented and unpigmented sheath samples, no obvious changes in sheath morphology were observed. Data from these two culture conditions have been reported separately, then averaged for use in comparisons with data generated by other authors. This approach, which may more accurately reflect sheath composition under field conditions, where the top millimeters are pigmented and shade the underlying non pigmented layers, is not without difficulties. Some parameters of sheath composition were more affected by the high-light regime than others. Amino acid composition was least affected, while total percent protein and total percent carbohydrate were lower in pigmented than in unpigmented sheath samples (Tables 1 and 2).

Scytonemin appears to play no role in sheath integrity. Its ease of removal indicates that it is not covalently bound to the carbohydrate. The unchanged sheath composition and integrity, with and without scytonemin, suggest that it plays no role in sheath structural chemistry. The sheath appears to play the role of a support matrix, allowing the pigment to be retained on the filament surface and thus to serve as a UV shield (Garcia-Pichel & Castenholz, 1991). Structural analysis of purified pigment by NMR identified two components, a monosubstituted phenol fragment and a disubstituted phenyl ring fragment (Alvi & Robbins, unpublished data). These data are in agreement with the full structure of scytonemin published by Proteau *et al.* (1993). This suggests that the pigment of *Lyngbya* is indeed scytonemin or a very similar pigment.

A number of questions about sheath architecture still remain. Among them are: the nature and function of glycoproteins within the sheath, the sequence of monosaccharides in the backbone and side of chains of sheath polysaccharide(s), the potential cross-linking of polymers to one another and what aspect of sheath structure confers resistance to degradation. These questions address sheath structure and function at a level above compositional analysis. However, the chemical resistance of the sheath precludes a

quick and simple solution. The groundwork on composition does point out the high degree of complexity found in the sheath of *L. aestuarii* and in cyanobacterial sheaths in general.

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